IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Richard A. Berg	Art Unit: 1819
Serial Nos.: 08/485,194	Examiner: B. Stanton
Filed: June 7, 1995	Docket No.: C94-007D1
Title: *Production of Human Recombinant Collagen in the Milk of Transgenic Animals*	Date: May 19, 1998
	Palo Alto, California

DECLARATION UNDER RULE 132

I, David P. Toman, declare and state as follows:

1. I am the Scientific Director of Molecular Biology research at Collagen Corporation, the assignee of the subject patent application. I am knowledgeable and experienced in the production of transgenes, transgenic animals and particularly, the expression of recombinant proteins in the milk of transgenic animals. I have read and am familiar with contents of the above application and the cited Meade et al., Barsh et al. and Ala-Kokko, et al. references. My curriculum vitae is attached.

2. The Meade reference suggests that a number of popularly recombinantly expressed proteins might be targeted for expression in mammalian milk. Meade's examples include coagulation factors VIII and IX, human or animal serum albumin, tissue plasminogen activator (TPA), urokinase, alpha-1 antitrypsin, animal growth hormones, Mullerian Inhibiting Substance (MIS), insulin, interferons, interleukins, milk lipases, antiviral proteins, peptide hormones, immunoglobulins and lipocortins (see Meade, column 3, lines 30-40), and exemplifies his method with bovine alpha S-1 casein expression.

3. There are a host of reasons why one would not have expected Meade's method to be amenable to mammary specific expression of human collagen. The collagen protein and gene present numerous differences from those suggested by Meade — differences which are associated with well-known obstacles to recombinant expression and secretion, and hence suggest the inapplicability of Meade's method. For examples, collagen is much larger and more rigid than the globular proteins suggested by Meade, collagen cDNA is much more GC rich than th cDNAs of those proteins suggested

by Meade and hence is prone to recombination and loss of gene fragments (see, e.g. Capello et al. (1996) US Pat No.5,496,712, esp. col 1, lines 59-66, enclosed), collagen is polymeric and requires multiple post-translational processing events (see, e.g. Davidson et al., 1981, Meth Enzym 23, 119-136, enclosed), and both collagen and procollagen are known to form intracellular aggregates (see, e.g. Trelstad et al., 1981, in Cell Biology of Extracellular Matrix, Plenum Press, p.179, esp.195-197, enclosed) and/or be diverted to degradation pathways (see, e.g. Berg, 1986, in Regulation of Matrix Accumulation, Acad.Press, p.29-52, esp. 36-46, enclosed). In fact, these attributes are so contraindicative of recombinant expression that, as of the effective filing date of the subject Application, collagen had never been expressed in a cell, such as a mammary cell, which did not already make a collagen (see e.g. Ala-Kokko et al., supra).

4. Furthermore, that a recombinant collagen could be expressed in vitro in a fibroblast already making collagen would not have suggested the feasibility of in vivo expression in mammary cells. For example, it was not known which of the many prostranslational processing events would be effected by the mammary cells (unlike fibroblasts which are known to have all the requisite machinery), whether the collagen secretory signal would effect proper vectorial secretion from the mammary cell (secretion if successful at all, might have been directed toward the basement membrane side of the cells), whether extracellular aggregation would prevent excretion from the aveolar ducts, etc. Accordingly, it is my opinion that one of ordinary skill in the art at the time of filing of the subject application, would not have had a reasonable expectation of successfully obtaining mammary specific expression of human collagen as instantly claimed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: 19 May 98

David P. Toman

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Richard A. Berg	Art Unit: 1819
Serial Nos.: 08/485,194	Examiner: B. Stanton
Filed: June 7, 1995	Docket No.: C94-007D1
Title: *Production of Human Recombinant Collagen in the Milk of Transgenic Animals*	Date: July 27, 1997
	Palo Alto, California

DECLARATION UNDER RULE 132

I, David P. Toman, declare and state as follows:

1. I am the Scientific Director of Molecular Biology research at Collagen Corporation, the assignee of the subject patent application. I am knowledgeable and experienced in the production of transgenes and transgenic animals. I have read and am familiar with contents of the above application. My curriculum vitae is attached.

2. The specification of the above reference application describes in experimental detail, the protocol used to generate a transgenic nonhuman mammal producing milk with human procollagen. The production and isolation from a human genomic library of a cosmid clone, CG103, containing the entire human α1(I) collagen gene is described in experimental detail (page 12, line 24-page 13, line 6). The importance of using genomic clones, as opposed to cDNA, is explained (page 13, lines 20-24). The specification describes a well-developed bovine system summarized by Krimpenfort, P. et al. (1991) in Biotechnology 9, 844-847, for expression of transgenes in milk exemplified with expression vectors comprising human genes fused to αS1-casein regulatory elements (page 10, lines 17-27). Technical details important for generating successful expression vectors such as ligation sites, insert size, promoter selection, etc. are set forth (page 13, line 29 - page 18, line 4). The isolation and transfection of host cells is described in experimental detail (page 18, line 5 - page 21, line 7). The isolation and purification of human procollagen form the milk of the resultant animals is described (page 21, line 22 - page 23, line 28).

3. Following the specification much like a recipe, my colleagues and I generated transgenic nonhuman mammals producing milk containing human procollagen as follows: (A) The human α1(I) procollagen gene was isolated from the clone CG103. See Figure 1, attached hereto. This step is described in the specification at page 12, line 24-page 13, line 6. (B) The structural gene for human α1(I) procollagen gene was fused with the bovine αS1-casein promoter. This step is described in the specification at page 16, line 20 and page 17, line 29. Murine oocytes were obtained, isolated and fertilized. These steps are described in the specification at page 18, lines 12-29. (D) The bovine αS1-casein promoter-human α1(I) procollagen gene fusion was microinjected into the fertilized murine oocytes to generate transgenic mice. This step is described in the specification at page 18, lines 5-12. (E) Transgenic mice were identified by Southern hybridization. This step is described in the specification at page 18, line 30-page 19, line 2. (F) As described in the specification at page 21, lines 22-30, the resultant mice yielded milk containing human procollagen. See Figure 2, attached hereto. Figure 2A shows that milk samples from our transgenic mice contained a new protein band which was the approximate size of the human α1(I) procollagen polypeptide chain. The identity of this polypeptide as human α1(I) procollagen was verified by its reaction with antiserum derived against a synthetic polypeptide fragment of the amino-terminus of human α1(I) procollagen (Figure 2B) and by susceptibility to bacterial collagenase (Figure 2C). Procollagen was recovered by separating the milk into discrete proteins by electrophoresis, excising the protein band which corresponded to human α1(I) procollagen in the corresponding immunoblot and eluting the human α1(I) procollagen from the gel. Approximately 10 μg of purified human α1(I) procollagen was recovered and subject to amino acid analysis which confirmed the identity of the recovered protein as human α1(I) procollagen.

4. Again following the specification much like a recipe, my colleagues and I generated transgenic rabbits producing milk containing human procollagen as follows: (A) The human α1(I) procollagen gene was isolated from the clone CG103. See Figure 1, attached hereto. This step is described in the specification at page 12, line 24-page 13, line 6. (B) The structural gene for human α1(I) procollagen gene was fused with the bovine αS1-casein promoter. This step is described in the

specification at page 16, line 20 and page 17, line 29. Rabbit oocytes were obtained, isolated and fertilized. These steps are described in the specification at page 18, lines 12-29. (D) The bovine αS1-casein promoter-human α1(I) procollagen gene fusion was microinjected into the fertilized rabbit oocytes to generate transgenic rabbits. This step is described in the specification at page 18, lines 5-12. (E) Transgenic rabbits were identified by Southern hybridization. This step is described in the specification at page 18, line 30-page 19, line 2. (F) As described in the specification at page 21, lines 22-30, the resultant rabbits yielded milk containing human procollagen. See Figure 3, attached hereto. Figure 3 (left gel) shows that milk samples from our transgenic rabbits contained a new protein band which was the approximate size of the human α1(I) procollagen polypeptide chain. The identity of this polypeptide as human α1(I) procollagen was verified by its reaction with antiserum derived against a synthetic polypeptide fragment of the amino-terminus of human α1(I) procollagen (Figure 3, right gel). Procollagen was recovered by separating the milk into discrete proteins by electrophoresis, excising the protein band which corresponded to human α1(I) procollagen in the corresponding immunoblot and eluting the human α1(I) procollagen from the gel. Approximately 10 μg of purified human α1(I) procollagen was recovered and subject to amino acid analysis which confirmed the identity of the recovered protein as human α1(I) procollagen.

5. In successfully carrying out the foregoing work, we followed the experimental outline provided by the subject patent application employing routine laboratory techniques known to those of ordinary skill in the art. The significant experimental methods were dictated by the teachings of the patent application; including, the selection of the structural collagen gene, the selection of the promoter, the generation of the transgene, the isolation of fertilized eggs, the transfection of such eggs and the implantation of the transfected eggs into female hosts. The resultant transgenic animals producing milk containing human procollagen was the predicted and, in fact, wholly expected result.

6. In my opinion, one of ordinary skill in this art could predictable generate a nonhuman transgenic animal producing milk containing human procollagen by following the guidelines of the subject patent application using only routine laboratory techniques known to those of ordinary skill in the art without undue experimentation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: July 28, 1997

David Toman

David P. Toman